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16. ABSTRACT

Genetic toxicants are present in polluted marine environments and may represent a long-term threat to populations of marine organisms. A cytogenetic model is useful to study the effects of these toxicants. The polychaete, *Neanthes arenaceodentata*, was chosen as such a model because it has a suitable karyotype, is easily cultured, and represents an ecologically important group of organisms. This paper presents details of an in vivo application of sister chromatid exchange (SCE) analysis, a sensitive cytogenetic technique, to this marine worm. In earlier studies, *N. arenaceodentata* exhibited a dose response to mitomycin C (MMC) at concentrations comparable to those that elicited responses in in vivo mammalian systems. Exposure to 5×10^{-7} M MMC for 48 h increased the frequency of SCE in the worm from a baseline value of 0.14 exchanges/chromosome to 0.5 exchanges/chromosome. Positive SCE responses in the worm have also been demonstrated in this study for other known, direct-acting mutagens such as 5-bromodeoxyuridine and methylmethanesulfonate, as well as for compounds that need metabolic activation such as benzo[*a*]pyrene, dimethylnitrosamine and cyclophosphamide. These results imply that *N. arenaceodentata* can metabolize promutagens and suggest that the worm may be sensitive to a broad spectrum of genetic toxicants. The significance of these findings, as well as directions for future research, are discussed.

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NEANTHES ARENACEODENTATA, A CYTOGENETIC MODEL FOR MARINE GENETIC TOXICOLOGY*

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Genetic toxicants are present in polluted marine environments and may represent a long-term threat to populations of marine organisms. A cytogenetic model is useful to study the effects of these toxicants. The polychaete, *Neanthes arenaceodentata*, was chosen as such a model because it has a suitable karyotype, is easily cultured, and represents an ecologically important group of organisms. This paper presents details of an in vivo application of sister chromatid exchange (SCE) analysis, a sensitive cytogenetic technique, to this marine worm. In earlier studies, *N. arenaceodentata* exhibited a dose response to mitomycin C (MMC) at concentrations comparable to those that elicited responses in in vivo mammalian systems. Exposure to 5×10^{-7} M MMC for 48 h increased the frequency of SCE in the worm from a baseline value of 0.14 exchanges/chromosome to 0.5 exchanges/chromosome. Positive SCE responses in the worm have also been demonstrated in this study for other known, direct-acting mutagens such as 5-bromodeoxyuridine and methylmethanesulfonate, as well as for compounds that need metabolic activation such as benzo[*a*]pyrene, dimethylnitrosamine and cyclophosphamide. These results imply that *N. arenaceodentata* can metabolize promutagens and suggest that the worm may be sensitive to a broad spectrum of genetic toxicants. The significance of these findings, as well as directions for future research, are discussed.

Key words: chromosome; genetic toxicants; polychaete; sister chromatid exchange; metabolic activation

INTRODUCTION

Pollutants in coastal marine environments may affect the genetic constitution of exposed organisms by either causing shifts in gene pool composition through selective mechanisms or by acting directly on the genetic material to produce mutations. This paper addresses the latter problem. There is evidence that some areas of the marine environment are contaminated with mutagens and carcinogens. Longwell and Hughes (1980) examined mackerel eggs sampled from the surface waters of the New York Bight. They observed mitotic-chromosome irregularities,

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variable development rates as calculated by mitotic index, and differences in viability as estimated by early indicators of cell death. These effects were found in the more impacted areas of the Bight close to the coast and dumping grounds. In addition to the New York Bight, mutagens have been detected in other polluted marine environments (Parry et al., 1976; Payne et al., 1979). These observations suggest that genetic toxicants may be present in many polluted marine environments and that genetic impairment may represent a long-term threat to populations of marine organisms.

Several approaches are possible to detect and study genetic toxicants. Chemical analyses of environmental samples can be performed, but many classes of compounds are genetically active so these analyses are time consuming and expensive. A simpler approach is to look for genetic damage in the exposed biota. Since cytogenetic techniques are sensitive and reasonably simple, it is recommended that genetic damage in marine organisms be determined by observing their chromosomes directly (IAEA, 1979; Kligerman, 1980).

Our goal was to develop an in vivo test so that whole organism responses could be studied and exposures could simulate complex environmental conditions. The success of this project depends on the choice of experimental animal and the selection of appropriate cytogenetic technique. A polychaete, *Neanthes arenaceodentata*, was selected for several reasons. First, it has a suitable karyotype, 18 relatively large chromosomes (Pesch and Pesch, 1980a). Second, it is easily handled and cultured, to provide a continuous supply of experimental material. Third, polychaetes are benthic and of particular interest because sediments are often reservoirs for pollutants. Fourth, they are important food web species for commercially important fishes, and therefore, may contribute to biomagnify toxicants. Fifth, they are relatively sedentary so field collected specimens would be representative of the area being sampled and finally, polychaetes are recognized as pollution indicators, particularly pollution associated with organic loading (Reish, 1960; Wass, 1967; Reish, 1972).

The cytogenetic technique of choice is metaphase chromosome analysis because observations of chromosomes are possible in a wide variety of tissues containing metaphase-arrested cells. Metaphase preparations also allow accurate observation of chromosome structure and permit sister chromatid exchange (SCE) analysis. Several studies have shown that SCE is a more sensitive method for detecting mutagens and carcinogens than the traditional chromosome and chromatid observations (Latt, 1974; Perry and Evans, 1975; Solomon and Bobrow, 1975; Bloom, 1978). The SCE technique also makes it possible to measure the effect of toxicants on cell replication kinetics because differential staining of chromosomes permits easy identification of first, second, third and subsequent replication cycle cells (Schneider et al., 1978).

We have previously presented details of an in vivo application of SCE analysis to the marine polychaete, *Neanthes arenaceodentata* (Pesch and Pesch, 1980b). The

present paper serves to update techniques and to present dose–response data for six known mutagens that include both direct-acting compounds and compounds that require metabolic activation.

MATERIALS AND METHODS

Neanthes arenaceodentata Moore (*Nereis acuminata* Ehlers, *Nereis caudata* Delle Chiaje) construct mucous tubes within sediment or on firm surfaces. Females deposit eggs within the mucous tubes, then die; males incubate the eggs, and larvae develop within the tubes. At 20 °C an entire life cycle (egg to egg) is completed in 9 to 10 wk. All of the *N. arenaceodentata* larvae used in this study were obtained from laboratory cultures that originated at California State University, Long Beach. Details of culture techniques have been published by Reish (1980).

For these experiments 10- to 12-day-old larvae were removed from the tubes and exposed in the dark to 5×10^{-5} M 5-bromodeoxyuridine in 50 ml of filtered sea water (30‰ salinity) for 48 h. Test compounds were mixed to appropriate concentrations with filtered sea water, then added concurrently with the BrdU. Colchicine (0.05%) was added to the sea water for the last 4 h of the 48-h exposure period. Mitomycin C (MMC), 5-bromodeoxyuridine (BrdU), and methylmethanesulfonate (MMS) were tested as examples of direct-acting compounds. Benzo[*a*]pyrene (B[*a*]P), dimethylnitrosamine (DMN) and cyclophosphamide (CP) were tested as examples of compounds that require metabolic activation. All compounds except B[*a*]P were water soluble and were added directly to sea water. B[*a*]P was initially dissolved in dimethylsulfoxide (DMSO). A solvent control (2% DMSO in sea water) was tested.

The following procedure for slide preparation was adapted from Kligerman and Bloom (1977). Sea water was decanted from each treatment and replaced with 100 ml of 0.075 M solution of potassium chloride for 1 h. Then the larvae were fixed in three changes of cold ethanol-acetic acid (3:1) for 0.5 h each. Fixed larvae (40–50) were placed in a clean well slide and 1 ml of 60% acetic acid was added to cover all the larvae. The larvae were macerated for approximately 1 min or until the tissue appeared translucent. The material was then drawn up into siliconized Pasteur pipettes and applied to clean, hot (45 °C) slides. Excess acetic acid was immediately removed from the slides. This procedure produced a monolayer of separated cells on each slide.

Slides were stained according to a procedure recommended by Bloom (pers. comm.). Slides with BrdU-labelled chromosomes were stained with 225 $\mu g \cdot ml^{-1}$ of 33258 Hoechst stain for 10 min (several drops placed on slide and coverslip added), rinsed in distilled water, and air dried. The slide was then wet mounted using an excess of McIlvaine's buffer (0.1 M citric acid, 0.2 M disodium phosphate) at pH 7.0 and placed between two black lights for 30 min. The slide was then rinsed in distilled water, air dried briefly, and stained with 3% Giemsa in McIlvaine's buffer

pH 7.0 (buffer diluted 1:1 with distilled water) for 10 min. The slide was rinsed in distilled water, air dried, soaked in xylene, and mounted in Coverbond. Observations were made with a research microscope at 1250X, oil immersion. Differential labelling with BrdU permitted easy identification of first-, second-, and third-division cells and the numbers of each were noted for every treatment condition. The proportion of second- and third-division cells was calculated. SCE observations were made on 25 second-division cells for each treatment unless otherwise noted. All cells observed were examined for gross chromosomal abnormalities. Photographs were taken at $1000\times$ through a green filter using high contrast copy film. Differences in the SCE data were subjected to analysis of variance and Duncan's multiple range test (Snedecor and Cochran, 1967), with $\alpha = 0.05$. Differences in the data for gross chromosomal abnormalities were tested for significance by a comparison of proportions test for binomial distributions, with $\alpha = 0.05$ (Snedecor and Cochran, 1967).

RESULTS

To obtain differentially stained chromosomes for SCE observations (Fig. 1), the DNA must be labelled with a base analog such as BrdU for two DNA replication cycles. However, BrdU is itself mutagenic and induces dose-dependent increases in SCE (Fig. 2). Therefore, it is important to define control or baseline conditions for SCE observations. For experiments reported here, control SCE frequencies were 4.1 ± 0.7/metaphase at 5×10^{-5} M BrdU. This compares favorably with a previous experiment where control SCE frequencies were 2.5 ± 1.3/metaphase at 3×10^{-5} M BrdU (Pesch and Pesch, 1980b).

All six compounds tested induced significant increases in SCE at some concentrations. Fig. 2 shows the responses for direct-acting compounds, Fig. 3 shows the responses for compounds that need metabolic activation. The frequency of SCEs for the DMSO solvent control did not differ from those for the standard control. Each data point for SCE in Fig. 2 is the mean of at least 25 metaphase counts with the exception of the points at the highest concentrations of MMC and MMS; these represent 8 and 9 counts, respectively. Each data point for SCE in Fig. 3 is the mean of at least 25 metaphase counts with the following exceptions: for B[*a*]P the counts for the three highest concentrations were 24, 18 and 21 with increasing concentrations, respectively; for CP, the counts from lowest to highest concentration were 25, 10, 12, 3, and 1, respectively.

The shapes of the SCE dose–response curves for direct-acting compounds were different from those for the compounds that need metabolic activation. The SCE responses for the direct-acting compounds (Fig. 2) increase with increasing dose except for the highest concentration of MMS. The SCE responses for the compounds that need metabolic activation were more complex. B[*a*]P and DMN exhibit what may be bimodal curves, while CP shows a general decline in SCE with

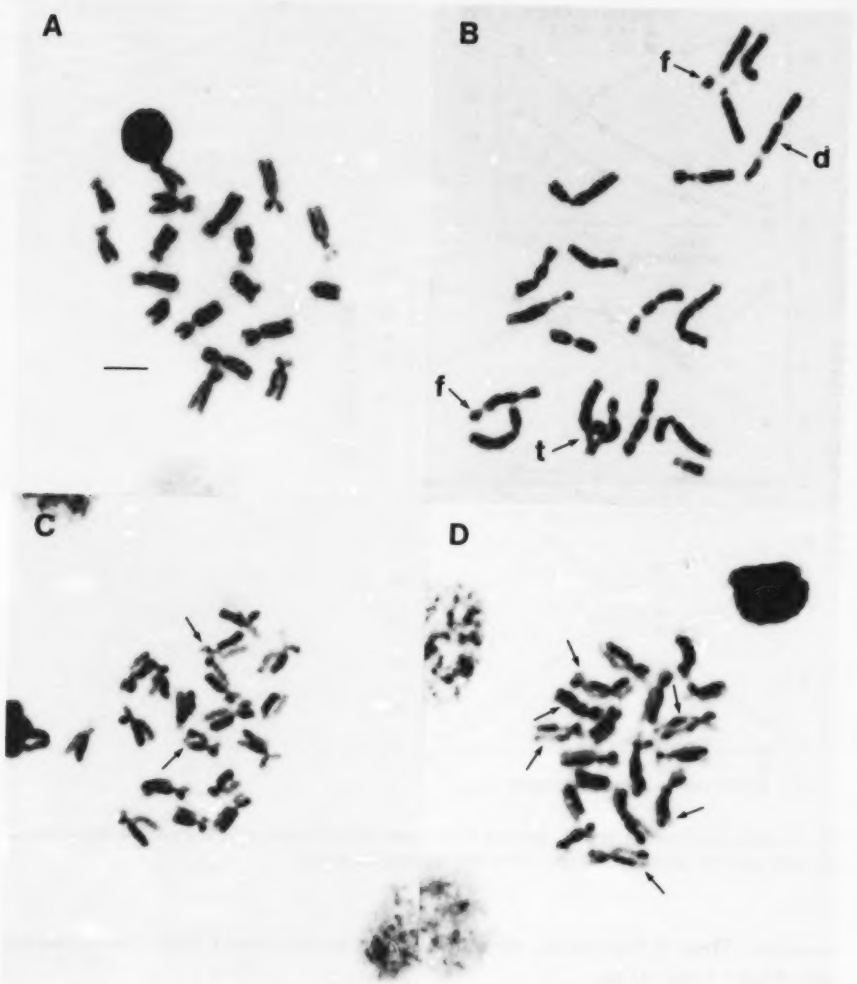

Fig. 1. Chromosomes of *N. arenaceodentata*. A, normal control, scale bar equals 5 μm; B, chromosomal aberrations, 5×10^{-5} M MMC (d = dicentric, f = fragment, t = translocation); C, differentially stained control; D, increases in SCEs, 1×10^{-5} M MMS.

increasing concentration. A bimodal curve may indicate that more than one mechanism of toxicity is influencing the SCE response. A decline in SCE response with increasing concentration is likely due to selective cell killing. In a heterogeneous population of cells, some cells are more sensitive to both killing and

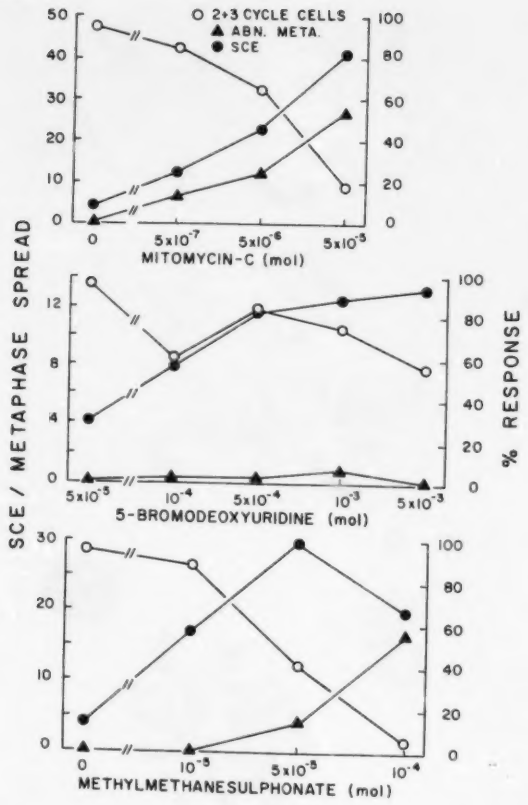

Fig. 2. Responses (number of SCE, percent second- and third-division cells, and percent chromosomal aberrations) of *N. arenaceodentata* to three direct-acting mutagens.

mutation. Thus, at high doses, the sensitive cells are destroyed, while the remaining cells exhibit fewer SCEs.

The relative proportion of second- and third-division cells to first-division cells for each treatment provides an indication of mitotic activity inhibition. Exposure time was selected empirically to maximize the proportion of second-division cells; therefore, the proportion of second- and third-division cells exceeds 95% in the controls. Five of the six compounds tested here (Figs. 2, 3) seem to inhibit mitotic activity at increased doses. The other compound, DMN had maximum effect of inhibition at an intermediate concentration.

All of the metaphase spreads were scored as either normal or abnormal. The abnormal designation meant that one or more chromosomes in the cell were

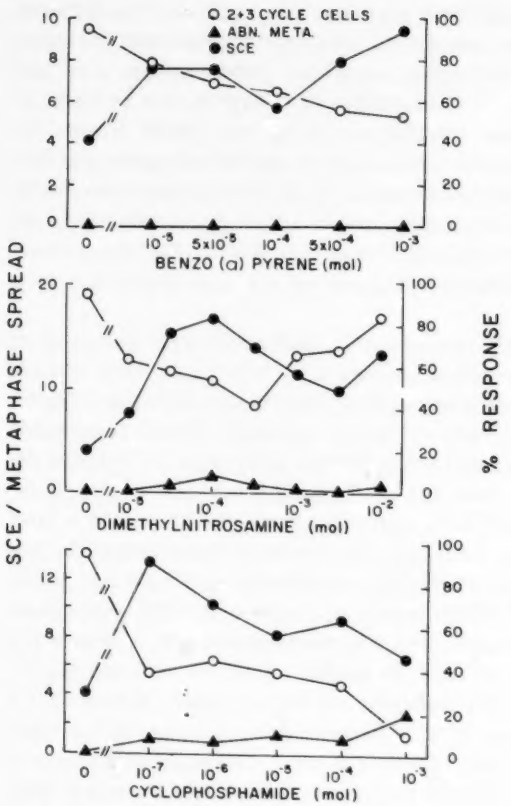

Fig. 3. Responses (number of SCE, percent second- and third-division cells, and percent chromosomal aberrations) of *N. arenaceodentata* to three mutagens that require metabolic activation.

distorted or broken. A representative example is seen in Fig. 1. All the compounds except B[*a*]P caused some gross abnormalities (Figs. 2, 3); however, only MMS, MMC and CP caused significant increases. The lack of significance for the BrdU and DMN data may have been due to the low cell sample sizes, which ranged from 21 to 207 with a mean of 70. Of 251 cells scored from B[*a*]P tested worms, none exhibited any chromosomal aberrations. Also, no chromosomal aberrations were observed in 44 cells from controls.

DISCUSSION

Exposure of *N. arenaceodentata* to B[*a*]P, CP, and DMN resulted in SCE

frequencies significantly higher than those associated with controls. This indicates that the worm can metabolize these compounds, a finding consistent with the report that polychaetes possess a mixed-function oxygenase (MFO) system (Lee and Singer, 1980; Lee et al., 1979). It is well established that B[*a*]P (review by Gelboin, 1980), CP (review by Mohn and Ellenberger, 1976), and DMN (review by Montesano and Bartsch, 1976) require metabolism to activate mutagens and that such transformations are mediated predominantly by the MFO (cytochrome *P*-450) system. Since most mutagens and carcinogens require metabolic activation in order to become either mutagenic or carcinogenic (Stetka and Wolff, 1976) these observations are important and suggest that *N. arenaceodentata* may be able to activate a broad spectrum of compounds.

Comparing the sensitivity of SCE response in *N. arenaceodentata* with other in vivo test systems is difficult because of differences in methodologies. Toxicants are administered by injections in most other studies. Despite this difference, similar doses of MMC to mice, rabbits, and *N. arenaceodentata* elicited comparable increases in SCE frequencies (Pesch and Pesch, 1980b). Kligerman (1979) tested the central mudminnow, *Umbri limi*, with two of the compounds used in this study, MMS and CP. Fish were injected, i.p., with 500 $\mu g \cdot g^{-1}$ BrdU and 24 h later injected, i.p., with a toxicant dose. Four days after exposure to a mutagen the fish were killed. A dose of 7 $\mu g \cdot g^{-1}$ MMS elicited a statistically significant increase in SCE frequency and 20 $\mu g \cdot g^{-1}$ MMS caused a doubling of SCE frequencies compared to controls. An even greater effect was observed with CP. A dose of 6.1 $\mu g \cdot g^{-1}$ CP caused a doubling of SCE frequency compared to controls. *N. arenaceodentata* were exposed to the mutagens via the sea water. A dose of 1.1 $\mu g \cdot ml^{-1}$ MMS caused an increase in SCE frequencies by a factor of 4.2 times control SCE frequencies, while a dose of 0.026 $\mu g \cdot ml^{-1}$ CP caused an increase in SCE frequencies by 3.2 times control SCE frequencies. Clearly, the worm is at least as responsive to mutagen induction of SCEs as is the central mudminnow.

The mechanism of SCE is not know. Theoretically, it represents a reciprocol exchange of identical DNA material between the two sister chromatids. SCE responses have been correlated with induced point mutations and may be useful as a quantitative indicator of mutagenesis (Carrano et al., 1978). There is considerable evidence suggesting that SCE is related to DNA repair. Many studies have found altered SCE responses in humans having DNA repair disorders (Chaganti et al., 1974; Latt et al., 1975; Wolff et al., 1977). SCE may represent more than one phenomenon; one type of SCE may be related to some form of DNA repair, while a second type of SCE may be related to DNA replication (Schneider et al., 1978). Although the exact mechanism of SCE is unknown, SCEs are believed to be a response to DNA damage since they can be induced by a wide variety of mutagens and carcinogens (Perry and Evans, 1975; Kligerman, 1977).

In order to understand the effects of genetic toxicants in marine environments, research projects should be designed to (1) determine the presence, distribution, and

abundance of mutagens in the marine environment, (2) assess the degree of genetic damage to marine organisms, and (3) ascertain the ecological consequences of such damage. No single test system can address all three concerns. Short-term tests are needed to identify mutagens, to bioassay complex mixtures and to assess acute responses of marine organisms. Long-term tests are needed to assess the effects of chronic exposures to individuals and to determine the consequences of these exposures to populations. A combination of field and laboratory approaches seems most useful. Field tests may serve as epidemiological tools to identify areas stressed by mutagens. However, field studies do not permit easy identification of cause–effect relationships. These relationships are best identified in well-structured laboratory experiments where, for example, dose-dependent responses may be demonstrated.

Our research goals include: standardizing and validating the short-term laboratory SCE test; developing long-term laboratory tests to investigate the effects of somatic and germinal mutations on populations; and finally, developing simple in situ tests for genetic toxicants in marine environments.

N. arenaceodentata will be used for the short- and long-term laboratory experiments. For in situ field testing, we intend to adapt cytogenetic techniques to other species more readily found in the field, for example, *Capitella capitata* and *Nereis virens*. As techniques are extended to other species, they will be applied to specimens collected from contaminated marine environments.

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